

**SEPARATE CYTOTOXIC T LYMPHOCYTE SUBSETS RECOGNIZE
THE DIFFERENT H-2 SPECIFICITIES***

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In mouse, the H-2.K, H-2.D, and H-2.L molecules encoded by the major histocompatibility complex bear the serologically defined private and public H-2 specificities (1, 2) and target determinants for alloreactive cytotoxic T lymphocytes (CTL) (3, 4). By studying the role of public specificities in cell-mediated reactions, Vazquez et al. (5) and others (6) found that the public specificities behave as targets for alloimmune CTL. In the next step of our work presented here, experiments were designed to investigate whether the different H-2 specificities are recognized by different subsets of CTL.

For several years, evidence has been accumulating that shows that the H-2.K, H-2.D, and H-2.L molecules are recognized by at least three independent subpopulations of CTL (4, 7, 8). Recently, by studying serologically identical H-2 mutants at the K^b locus, Geib et al. (9) have shown that different antigenic determinants are responsible for the generation of multiple CTL clones within one H-2 molecule. Our data, based on specific removal of alloimmune CTL on relevant macrophage monolayers provide evidence that an allogeneic stimulation induces the generation of different subsets of CTL that are able to recognize separately the private and the public specificities of one H-2 molecule and are able to discriminate between the different public specificities themselves.

Materials and Methods

Mice. 2- to 3-mo-old mice of both sexes listed in the tables were kindly provided by Professors J. Colombani and J. P. Levy (Service National Commun 7, Institut National de la Santé et de la Recherche Médicale, Paris, France). H-2^{dm2} mutant mice were a gift of Dr. P. Démant (Netherlands Cancer Institute, Amsterdam, Netherlands).

Source of Alloreactive CTL. Spleen cells were obtained from mice once primed in vivo and restimulated 11 d later in vitro with the same allogeneic cells as described previously (5).

Adsorption of Alloreactive CTL on Macrophage Monolayers. The procedure was based on that described previously (7, 10). Mouse peritoneal macrophages (2×10^7 cells in 5 ml medium), harvested 2 d after intraperitoneal injection of 2 ml of thioglycollate medium (Difco Laboratories, Detroit, Mich.), were seeded in 25-cm³ tissue culture Falcon flasks (3013; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) to obtain a uniform cell monolayer, and were incubated for 24-48 h at 37°C. Alloimmune spleen cells (10^7 cells in 2 ml medium) were then placed on the prewashed monolayers. Adsorption was carried out for 90 min at 37°C, and was followed by the recovery of the nonadherent cells which contained <1% detached macrophages as seen by morphological criteria.

Cytotoxicity Assay. Target cells were phytohemagglutinin (PHA)-stimulated spleen cells

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(1 $\mu\text{g}/\text{ml}$ of PHA, Wellcome Research Laboratories, Beckenham, Kent, England). 2×10^4 target cells, ^{51}Cr - (Commissariat à l'Énergie Atomique, Saclay, France) labeled, were incubated with 1×10^6 effector cells in 200 μl of culture medium for 3.5 h at 37°C (5). Specific ^{51}Cr release was calculated as: $(E - C)/(T - C) \times 100$, where E is the release in presence of immune spleen cells, C the release in presence of unstimulated spleen cells, and T the maximum release upon addition of 4 N HCl. Spontaneous release did not exceed 20–30% of the total release.

Results and Discussion

To investigate if different subsets of alloreactive CTL react against the public and private H-2 specificities expressed on one H-2 molecule, C3H (H-2^k) anti-C3H.OL (K^dI^dD^k) CTL were adsorbed on a mixture of macrophages from C3H.Q and C3H.B10 strains that expressed the whole set of sensitizing public H-2 specificities (11). The residual cytotoxicity left in the nonadherent cell population was then tested on C3H.OL target cells. As shown in Table I, this adsorption was followed by a persistent, although slightly decreased, cytotoxicity against C3H.OL target cells (62–53% of lysis), whereas the same adsorption completely removed the cross-killing directed against C3H.Q and C3H.B10 targets and also against B10.A which share the H-2.28, 29 public specificities with the latter strains. Adsorption of the CTL on control C3H monolayers did not significantly decrease their lytic capacity, indicating that the adsorption was specific. In contrast, their adsorption on C3H.OL monolayers did not leave residual cytotoxicity on any target, showing the efficiency of the adsorption procedure. Therefore, the lack of cross-killing of C3H.Q, C3H.B10, and B10.A targets resulting from adsorption on C3H.Q and C3H.B10 suggests the existence of a subpopulation of CTL specific for the public specificities expressed on the H-2.K^d molecule. On the other hand, the strong lysis still observed on C3H.OL after this adsorption, argues for the existence of one or several CTL subsets specific for the H-2.31 private specificity expressed on the H-2.K^d molecule.

One could argue that the killing exerted by the nonadherent population is in fact directed against other antigens than H-2, namely, Ia, and/or T-region antigens (12–14). As for Ia antigens, several arguments indicate that, in our experimental conditions, it is likely that they do not act as target determinants because (a) the target cells were PHA blasts which express few Ia antigens (15) and (b) no lysis was found on B10.A target cells expressing the Ia 6 sensitizing specificity absent on C3H.Q and C3H.B10 macrophages used for adsorption. As for T-region antigens, previous studies by Vazquez et al. (5) have shown that they are not significantly involved in the cross-killing exerted against public specificities. Furthermore the killing exerted by the anti-private subset cannot be explained by the contamination of CTL reacting against T-region antigens, because B10 targets, expressing the Q_a and H-2.T “a” antigens (11) were not killed, although effector cells (C3H) were Q_a^b. In conclusion, the first set of experiments shows that subsets of CTL specific for public H-2 specificities can be separated from other subsets specific for the private specificities expressed on the same molecule.

In a next set of experiments the cross-reacting cytolytic activity of B10.BR (H-2^k) anti-B10 (H-2^b) CTL was tested on B10.AKM target cells after adsorption on B10.D2 macrophages to see if the public specificities are recognized by different subsets of CTL. As shown in Table II, a low but significant lysis (10%, $P < 0.001$) was left on B10.AKM targets where the public specificity H-2.56 could be theoretically detected (11). Likewise, adsorption on B10.AKM left significant lysis on: (a) B10.D2 cells (18%)

TABLE I
Adsorption of C3H Anti-C3H.OL CTL on Macrophage Monolayers

Adsorption monolayers	Target cells				
	C3H (k kkkkk k)*	C3H.OL (d ddddd k)*	C3H.Q (q qqqqq q)*	C3H.B10 (b bbbbb b)*	B10.A (k kkkkd d)*
—	0.1 ± 0.5	65 ± 2.1	21 ± 1.2	25 ± 1.4	21.5 ± 0.7
C3H	2 ± 1.2	62 ± 3.1 <i>H-2.31, H-2.28, 29, 34, 46</i>	19 ± 0.7 <i>H-2.28, 29, 34</i>	23 ± 0.8 <i>H-2.28, 29, 46</i>	20 ± 1.3 <i>H-2.28, 29</i>
C3H.OL	1.2 ± 0.7	8.4 ± 1.2	2.1 ± 0.9	3.4 ± 1.1	4.2 ± 0.6
C3H.Q + C3H.B10	0.1 ± 0.4	53 ± 2.4 <i>H-2.31</i>	3.7 ± 0.7	4.2 ± 0.7	6 ± 1.2

Data are the mean values of triplicates ± SD. The H-2 specificities theoretically recognized by the nonadherent subsets are given in italics. (The private specificity is in boldface italics). B10.A strain is Qa1,2,3^a, H-2.T^a; C3H: Qa1,2,3^b, H-2.T(?); C3H and C3H.OL are identical in the T1a region.

* Alleles for H-2.K, IA, IB, IJ, IE, IC, ID, and H-2.D regions and subregions.

where specificities H-2.35, 36, and 46 could be recognized and on: (b) C3H.OL cells (10%, $P < 0.001$) corresponding to specificity H-2.46. After adsorption on C3H.OL monolayers that expressed only the sensitizing public specificities H-2, 28, 29, and 46, the CTL could still lyse B10.D2 (19%) and B10.AKM cells (18%) that expressed other stimulating public specificities (11). Interference of the I-region products could be ruled out in all experiments by the use of PHA blasts as target cells (see above) and, for genetic reasons, on B10.AKM target cells that share the whole I region with the effector cells (B10.BR). That the lysis exerted by the nonadherent subsets could be a result of CTL reacting against Qa2,3 antigens, the only T-region antigens which may be recognized in these combinations (11), can also be ruled out. For instance, the CTL adsorbed on B10.D2 macrophages still killed B10.AKM and not B10.D2 target cells, although both expressed the Qa2,3^a antigens. In conclusion, the data shown in Table II suggest that public specificities themselves are recognized by different subpopulations of CTL.

This conclusion was supported by another set of experiments (Table III) in which B10.BR (H-2^k) anti-B10.A (K^kI^k/d^d) CTL were used. Their adsorption on B10.AKM monolayers still allowed a residual cytotoxicity on B10 targets (13%, $P < 0.01$) were public specificities H-2.35, 36 could be detected according to the H-2 chart (11). Furthermore, in this semi-alloreactive combination, CTL specifically reacting against H-2.L^d products were generated as previously suggested by blocking experiments (4), such CTL could be separated after adsorption on H-2^{dm2} mutant macrophages (identical to BALB/c except for the loss mutation on the H-2.L locus), as indicated by the residual cytotoxicity (23%) left on B10.A targets. A significant lysis was also observed on B10.AKM targets (12%) but not on B10 (4.5%). These data are in accordance with the stronger cross-reaction, described at the serological and CML levels, between H-2.L^d and H-2.L^a molecules as compared to H-2.L^d and H-2.L^b (2, 16). Surprisingly, adsorption of the CTL on B10 or B10.AKM targets (H-2.28, 29 positive) did not remove the cytotoxicity on C3H.OL targets (10%, $P < 0.01$) that also express the H-2.28, 29 specificities. Because it is improbable that the killing may be directed against Ia and Qa2,3 antigens (see above and Table III), this persistent lysis might be a result of the detection, by CTL subsets, of the H-2 allele-associated

TABLE II
Adsorption of B10.BR Anti-B10 CTL on Macrophage Monolayers

Adsorption monolayers	Target cells				
	B10.BR (k kkkkk k)*	B10 (b bbbbb b)*	B10.D2 (d ddddd d)*	B10.AKM (k kkkkk q)*	C3H.OL (d ddddd k)*
—	2.3 ± 0.5	74 ± 2.3	40 ± 1.2	42 ± 0.5	41 ± 1.4
B10.BR	3 ± 0.8	72 ± 3.1	38 ± 1.4 <i>H-2.6, 27, 28, 29, 35, 36, 46</i>	39 ± 2.1 <i>H-2.6, 27, 28, 29, 56</i>	37 ± 2.4 <i>H-2.6, 28, 29, 46</i>
B10	2 ± 1.1	9.2 ± 1.1	3.5 ± 0.9	2.8 ± 1.1	3.9 ± 1.3
B10.D2	3 ± 0.7	60 ± 2.3	3 ± 1.2	10 ± 0.9‡ <i>H-2.56</i>	4 ± 1.1
B10.AKM	2 ± 1.3	62 ± 1.8	18 ± 1.2‡ <i>H-2.35, 36, 46</i>	4 ± 0.6	10 ± 0.7‡ <i>H-2.46</i>
C3H.OL	0.5 ± 1.1	60 ± 0.9	19 ± 2.2‡ <i>H-2.6, 27, 35, 36</i>	18 ± 1.9‡ <i>H-2.6, 27, 56</i>	2.4 ± 0.9

Data are the mean values of triplicates ± SD. The H-2 public specificities recognized on the cross-reacting targets by the nonadherent subsets are in italics. B10.BR is Qa1^a, Qa2.3^b, H-2.T^a, Tla^a; B10: Qa1^b, Qa2.3^a, H-2.T^a, Tla^b; B10.D2: Qa1^a, Qa2.3^a, H-2.T^a, Tla^a; B10.AKM: Qa1^a, Qa2.3^a, H-2.T (?), Tla^a; C3H.OL: Qa1 (?), Qa2.3 (?), H-2.T (?), Tla^b.

* Alleles at the H-2K, IA, IB, IJ, IE, IC, and H-2.D regions and subregions.

‡ Significantly more than when tested on target cell syngeneic with macrophage monolayer ($P < 0.001$).

TABLE III
Adsorption of B10.BR Anti-B10.A CTL on Macrophage Monolayers

Adsorption monolayers	Target cells				
	B10.BR (k kkkkk k)*	B10.A (k kkkkd d)*	B10 (b bbbbb b)*	C3H.OL (d ddddd k)*	B10.AKM (k kkkkk q)*
—	1.5 ± 1.2	62 ± 3.1	23 ± 1.2	21 ± 2.1	32 ± 1.9
B10.BR	2.2 ± 0.9	60 ± 2.9	20 ± 1.7	19.8 ± 1.8	31 ± 0.6
B10.A	3.1 ± 2.1	9.3 ± 1.2	2.2 ± 0.9	2.1 ± 1.2	3.5 ± 1.7
B10	2 ± 0.9	55 ± 2.1	4.5 ± 0.7	10 ± 1.1§	17 ± 0.5 <i>H-2.13, 65</i>
C3H.OL	1.5 ± 1.1	55 ± 0.9	15 ± 0.8 <i>H-2.6, 27, 35, 36, 64</i>	3 ± 0.7	19 ± 0.9 <i>H-2.6, 13, 27, 64, 65</i>
B10.AKM	2 ± 0.7	51 ± 1.5	13 ± 1.1§ <i>H-2.35, 36</i>	9 ± 1.5§	4 ± 0.8
H-2 ^{dm2} ‡	2.3 ± 1.1	24 ± 1.7§ <i>H-2.64, 65</i>	4.5 ± 0.9 <i>H-2.64</i>	2.6 ± 1.2	11.8 ± 1.1§ <i>H-2.64, 65</i>

Data are the mean values of triplicate ± SD. The H-2 public specificities recognized on the cross-reacting targets by the nonadherent subsets are in italics. B10.BR and B10.A are identical in their Qa1, H-2.T loci and Tla region. B10.A, B10, and B10.AKM are Qa2.3^a.

* Alleles at the H-2K, IA, IB, IJ, IE, IC, and H-2.D regions and subregions.

‡ H-2.L loss mutant of BALB/c. The lysis of H-2^{dm2} targets after this adsorption was 2.5 ± 1.3.

§, || Significantly more than when tested on target cells syngeneic with macrophage monolayers (§ $P < 0.001$; || $P < 0.001$).

polymorphism of the specificities of the H-2.28 family (1, 2) or of nonserologically detectable H-2 determinants (17).

In conclusion, these results indicate that an allogeneic stimulation induces the development of several subpopulations of CTL which discriminate between: (a) the private and public specificities expressed on one H-2 molecule and (b) the public

specificities themselves. In all experiments, adsorptions of the CTL on cross-reacting monolayers decreased the lysis on target cells where they should recognize H-2 specificities. One obvious explanation is that, if all the CTL are unipotential, adsorption removes part of them, therefore lowering the real number of effector cells per target cell. The existence of other subsets of T cells that recognize several H-2 specificities altogether can also explain such a phenomenon. Analysis of our data show that although in most cases the residual cytotoxicity exerted by the nonadherent subsets of CTL follows the presence or absence of public specificities, in two cases (Table III, C3H.OL targets) residual cytotoxicity was observed, although no public specificities should have been detected. This finding is in accordance with the fact that CTL can be developed between serologically identical H-2 mutants, suggesting that other determinants than those recognized by alloantibodies could act as targets for CTL (18). However, it has been recently suggested that antibodies could be raised between such mutants by using appropriate immunization (18).

One of the major characteristics of the H-2 antigens is their polymorphism which is related to the expression of many different specificities on each H-2 molecule (1). On the basis of the results presented here, one may speculate that, if each specificity is recognized by a separate clone of lymphocytes, an allogeneic stimulation must generate a large number of T cell clones in response to the multiple H-2 determinants. Such a high number of T cell clones would be in accordance with the remarkable high frequency of CTL for a foreign H-2 haplotype described at the precursor level (19).

Summary

Using a monolayer adsorption technique, the fine specificity of cytotoxic effector T lymphocytes (CTL) generated against allogeneic or semi-allogeneic H-2 haplotypes was investigated. The results show that: (a) CTL reacting with the private specificity expressed on an H-2.K molecule can be separated from those reacting with the public specificities expressed on the same molecule and (b) the CTL that recognize cross-reacting H-2 determinants (public specificities) can also be separated into several subpopulations. These data support the hypothesis that an allogeneic stimulation induces a large number of independent T cell clones that react with H-2 determinants.

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